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Project Title: Genetic and chemical intervention in ROS signaling pathways affecting development and pathogenicity of *Sclerotinia sclerotiorum*

Investigators	Institutions
Principal Investigator (PI): Martin B. Dickman	Texas A&M Univ.
Co-Principal Investigator (Co-PI): Oded Yarden	The Hebrew Univ. of Jerusalem
Collaborating Investigators:	

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
Catalase, Fungal virulence, Oxidative stress, Necrotroph, Programmed cell death,

Abbreviations commonly used in the report, in alphabetical order:

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Jane Zuber
Director, Contracts and Grants
Texas A&M University System

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	3	13	0	16
Submitted, in review, in preparation		2		2
Invited review papers		3		3
Book chapters		1		1
Books				
Master theses	2			
Ph.D. theses	1			
Abstracts	5	6		11
Not refereed (proceedings, reports, etc.)				0

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Dr. Brett Williams –Queensland Institute of Technology-Assistant Proesor

Dr. Mehdi Kabbage-University of Wisconsin-Assistant Professor

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1		2
Longer Visits (Sabbaticals)				

Abstract: The long-term goals of our research are to understand the regulation of sclerotial development and pathogenicity in *S. sclerotiorum*. The focus in this project was on the elucidation of the signaling events and environmental cues involved in the regulation of these processes, utilizing and continuously developing tools our research groups have established and/or adapted for analysis of *S. sclerotiorum*.

Our stated objectives: To take advantage of the recent conceptual (ROS/PPs signaling) and technical (amenability of *S. sclerotiorum* to manipulations coupled with chemical genomics and next generation sequencing) developments to address and extend our fundamental and potentially applicable knowledge of the following questions concerning the involvement of REDOX signaling and protein dephosphorylation in the regulation of hyphal/sclerotial development and pathogenicity of *S. sclerotiorum*: (i) How do defects in genes involved in ROS signaling affect *S. sclerotiorum* development and pathogenicity? (ii) In what manner do phosphotyrosine phosphatases affect *S. sclerotiorum* development and pathogenicity and how are they linked with ROS and other signaling pathways? And (iii) What is the nature of activity of newly identified compounds that affect *S. sclerotiorum* growth? What are the fungal targets and do they interfere with ROS signaling?

We have met a significant portion of the specific goals set in our research project. Much of our work has been published. Briefly, we can summarize that: (a) Silencing of SsNox1 (NADPH oxidase) expression indicated a central role for this enzyme in both virulence and pathogenic development, while inactivation of the SsNox2 gene resulted in limited sclerotial development, but the organism remained fully pathogenic. (b) A catalase gene (*Scat1*), whose expression was highly induced during host infection is involved in hyphal growth, branching, sclerotia formation and infection. (c) Protein tyrosine phosphatase 1 (*ptp1*) is required for sclerotial development and is involved in fungal infection. (d) Deletion of a superoxide dismutase gene (*Sssod1*) significantly reduced in virulence on both tomato and tobacco plants yet pathogenicity was mostly restored following supplementation with oxalate. (e) We have participated in comparative genome sequence analysis of *S. sclerotiorum* and *B. cinerea*. (f) *S. sclerotiorum* exhibits a potential switch between biotrophic and necrotrophic lifestyles (g) During plant-microbe interactions cell death can occur in both resistant and susceptible events. Non-pathogenic fungal mutants *S. sclerotiorum* also cause a cell death but with opposing results. We investigated PCD in more detail and showed that, although PCD occurs in both circumstances they exhibit distinctly different features. The mutants trigger a restricted cell death phenotype in the host that unexpectedly exhibits markers associated with the plant hypersensitive (resistant) response. Using electron and fluorescence microscopy, chemical effectors and reverse genetics, we have established that this restricted cell death is autophagic. Inhibition of autophagy rescued the non-pathogenic mutant phenotype. These findings indicate that autophagy is a defense response in this interaction Thus the control of cell death, dictated by the plant (autophagy) or the fungus (apoptosis), is decisive to the outcome of certain plant-microbe interactions. In addition to the time and efforts invested towards reaching the specific goals mentioned, both PIs have initiated utilizing (as stated as an objective in our proposal) state of the art RNA-seq tools in order to harness this technology for the study of *S. sclerotiorum*.

The PIs have met twice (in Israel and in the US), in order to discuss and coordinate the research efforts. This included a working visit at the US PIs laboratory for performing RNA-seq experiments and data analysis as well as working on a joint publication (now published).

The work we have performed expands our understanding of the fundamental biology (developmental and pathogenic) of *S. sclerotiorum*. Furthermore, based on our results we have now reached the conclusion that this fungus is not a bona fide necrotroph, but can also display a biotrophic lifestyle at the early phases of infection. The data obtained can eventually serve a basis of rational intervention with the disease cycle of this pathogen.

Achievements

The *Sclerotinia sclerotiorum* NADPH Oxidases (Ssnox1, Ssnox2). We identified two *S. sclerotiorum* NADPH oxidases (*SsNox1/2*). Silencing of *SsNox1* expression indicated a central role for this enzyme in both virulence and pathogenic (sclerotial) development, while inactivation of the *SsNox2* gene resulted in limited sclerotial development, but the organism remained fully pathogenic. Δ*Ssnox1* strains had reduced ROS levels, did not develop sclerotia, and unexpectedly correlated with reduced oxalate production.

The *Sclerotinia sclerotiorum* catalase Scat1. Catalases play a role in the detoxification of ROS by converting H_2O_2 to water and molecular oxygen. Moreover, H_2O_2 is involved in the cross-linking of cell wall appositions to physically block pathogen penetration through induction of defense-related genes. We identified and functionally characterized the single copy type A catalase gene (designated *Scat1*), whose expression was highly induced during host infection. Δ*Scat1* mutants displayed reduced hyphal growth which was accompanied by hyperbranching and delayed, abnormal, sclerotia formation. Loss of *Scat1* attenuated fungal infection and rendered these mutants hypersensitive to SDS, osmotic and salt stresses. Δ*Scat1* exhibited resistance to the polyene ergosterol inhibitors amphotericin-B and nystatin and Δ*Scat1* was found to have a 5-fold increase in ergosterol content. Our results suggest that SCAT1 is involved in ROS-related activities associated with hyphal integrity, altered ergosterol levels, and defects in pathogenic development.

The *Sclerotinia sclerotiorum* superoxide dismutase (Sssod1) We have identified a *S. sclerotiorum* SOD (*Sssod1*) with high similarity to CuZnSODs. Treatment with the CuZnSOD inhibitor diethyldithiocarbamate (DETC) resulted in delayed hyphal growth and sclerotial development in a dose-dependent manner. Δ*Sssod1* mutants exhibited morphological defects similar to those observed with the inhibitor treatment. Moreover, Δ*Sssod1* was more sensitive than wild-type to menadione, a redox cycling agent. Expression of *Sssod1* was induced following treatment with oxidizing agents. The Δ*Sssod1* mutant was significantly reduced in virulence on both tomato and tobacco plants compared to wild-type In accordance with reduced virulence, Δ*Sssod1*induced a host oxidative burst in adjacent uninfected cells, a phenotype indicative of active pathogen recognition by the host. Intriguingly, during wild-type infection, host ROS production was significantly reduced. These results suggest that wild-type *Sclerotinia* suppresses host defense responses during infection.

We have also been involved in a community-based project in which the **genome of *S. sclerotiorum* has been deciphered** (in comparison with the related fungus - *Botrytis cinerea*). Their 38–39 Mb genomes include 11,860–14,270 predicted genes, which share 83% amino

acid identity between the two species. Genome organization analysis revealed large scale co-linearity between the two fungi. The arsenal of genes associated with necrotrophic processes is similar between the species. Analysis of secondary metabolism gene clusters revealed an expansion in number and diversity of *B. cinerea*–specific secondary metabolites relative to *S. sclerotiorum*, suggesting adaptation to specific ecological niches. Comparative genome analysis revealed the basis of differing sexual mating compatibility systems between *S. sclerotiorum* and *B. cinerea*. The organization of the mating-type loci differs, and their structures provide evidence for the evolution of heterothallism from homothallism.

***Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment and controlling cell death** Our recent work indicated that oxalic acid (OA), produced by *S. sclerotiorum*, can induce apoptotic-like programmed cell death (PCD) in plant hosts. The induction of PCD and disease requires generation of ROS in the host, a process triggered by fungal secreted OA. Conversely, during the initial stages of infection, OA also dampens the plant oxidative burst, an early host response generally associated with plant defense. This scenario presents a challenge regarding the mechanistic details of OA function; as OA both suppresses and induces host ROS during the compatible interaction. We generated transgenic plants expressing a redox-regulated GFP reporter. Results show that initially, Sclerotinia (via OA) generates a reducing environment in host cells that suppress host defense responses including the oxidative burst and callose deposition, akin to compatible biotrophic pathogens. Once infection is established however, this necrotroph induces the generation of plant ROS leading to PCD of host tissue, the result of which is of direct benefit to the pathogen. In contrast, a non-pathogenic OA-deficient mutant failed to alter host redox status. The mutant produced hypersensitive response-like features following host inoculation, including ROS induction, callose formation, restricted growth and cell death. These results indicate active recognition of the mutant and further point to suppression of defenses by the wild type necrotrophic fungus. Chemical reduction of host cells with dithiothreitol (DTT) or potassium oxalate (KOA) restored the ability of this mutant to cause disease. Moreover, recently we have established two mechanistically distinct forms of PCD (apoptosis and autophagy). Thus, *Sclerotinia* uses a novel strategy involving regulation of host redox status to control cell death and establish infection. These results address a long-standing issue involving the ability of OA to both inhibit and promote ROS to achieve pathogenic success.

Cell death control: The interplay of apoptosis and autophagy in the pathogenicity of *Sclerotinia sclerotiorum* PCD is characterized by a cascade of tightly controlled events that culminate in the orchestrated death of the cell. In multicellular organisms autophagy and

apoptosis are recognized as two principal means by which these genetically determined cell deaths occur. During plant-microbe interactions cell death programs can mediate both resistant and susceptible events. Via oxalic acid (OA), *S. sclerotiorum* hijacks host pathways and induces cell death in host plant tissue resulting in hallmark apoptotic features in a time and dose dependent manner. OA-deficient mutants are non-pathogenic and trigger a restricted cell death phenotype in the host that unexpectedly exhibits markers associated with the plant hypersensitive response including callose deposition and a pronounced oxidative burst, suggesting the plant can recognize and in this case respond, defensively. The details of this plant directed restrictive cell death associated with OA deficient mutants is the focus of this work. Using a combination of electron and fluorescence microscopy, chemical effectors and reverse genetics, we show that this restricted cell death is autophagic. Inhibition of autophagy rescued the non-pathogenic mutant phenotype. These findings indicate that autophagy is a defense response in this necrotrophic fungus/plant interaction and suggest a novel function associated with OA; namely, the suppression of autophagy. These data suggest that not all cell deaths are equivalent, and though programmed cell death occurs in both situations, the outcome is predicated on who is in control of the cell death machinery. Based on our data, we suggest that it is not cell death per se that dictates the outcome.

Over the course of this project and based on new data emerging from our labs, we have revisited the current line of thought, in which *S. sclerotiorum* is considered a classic necrotroph, and have suggested that it should be redefined as a hemibiotroph. **The potential switch between biotrophic and necrotrophic lifestyles of *S. sclerotiorum*** have been summarized in a recent co-authored review. Rather than overwhelming plant foes, *S. sclerotiorum* has evolved clever means to compromise host recognition and establish disease, resulting in a broad and immensely successful pathogenic lifestyle. We propose that the hemi-biotrophic lifestyle may be more temporally and spatially complex than currently depicted, and that combining lifestyle attributes with damage response curves that consider the contribution of both the fungus and the host to pathogenesis, may provide a more holistic manner to view plant pathogens.

Details of cooperation

The cooperation between the two PIs was continuous and supportive. This included sharing of information as well as materials (strains) throughout the project. It also included respective visits to the partners labs. In the case of the Yarden visit to Texas A&M, it also included actual work on RNASeq of *S. sclerotiorum* samples, taking advantage of the local infrastructure. Furthermore, during both visits, the PIs spent significant time in brainstorming, coordinating continued work, meetings with students as well as summarizing our research (in part, in the form of joint publications). Our collaboration has exceeded the foci of this project and have expanded to planning future collaboration as well as joint teaching in our respective plant pathology academic programs. Dickman taught an advanced course class session in Israel and Yarden taught an advanced plant pathology class, via skype, to Texas A&M graduate students.